AWARD NUMBER: W81XWH-15-2-0066

TITLE: A Nanolayer Copper Coating for Prevention of Nosocomial Multi-drug Resistant Infections

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REPORT DATE: December 2017

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Decemebr 2017	Final	15 Sep 2015 - 14 Sep 2017

4. TITLE AND SUBTITLE
A Nanolayer Copper Coating for Prevention of Nosocomial Multi-Drug Resistant Infections

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-2-0066

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER
8

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project aims to establish an antimicrobial and biocompatible copper coating for a standard issue antimicrobial combat wound dressing. This initial one-year project was done to provide important foundational data that will guide subsequent research efforts and position the technology for commercial development. As part of these efforts, iFyber established a number of coating parameters a number of candidate dressings that have a range of *in vitro* antimicrobial efficacy and biocompatibility performance metrics. Best performing candidates were shown to be biocompatible through an *in vivo* sensitization study and effective against a number of clinical bacterial isolates.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	23	USAMRMC

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1.0 Introduction

The long-term goal of this project is to establish an enabling antimicrobial technology aimed at providing a range of anti-infective materials for use by the military. To accomplish this goal, iFyber's immediate research and development centered on identifying and optimizing an antimicrobial and biocompatible copper coating for a standard issue antimicrobial combat wound dressing. This initial one-year project was done to provide important foundational data that will guide subsequent research efforts and position the technology for commercial development. As part of these efforts, iFyber established a number of coating parameters to produce a range of candidate dressings for evaluation in *in vitro* antimicrobial efficacy assays and biocompatibility assays using mammalian cells. Best performing candidates were then taken on to *in vivo* biocompatibility studies to foundational data regarding the safety of the copper coating. This annual report details the major accomplishments of this study, and outlines next steps in the development of an antimicrobial combat wound dressing.

2.0 Keywords

antimicrobial, copper, cotton, wound dressing, biocompatibility

3.0 Accomplishments

3.1 Goals of the Project

The long-term goal of this effort is to establish an enabling antimicrobial technology aimed at providing a range of anti-infective materials for use by the military. iFyber's immediate research and development goals set forth in the current effort are centered on identifying and optimizing an antimicrobial and biocompatible copper coating for a standard issue wound dressing. Below is a list of technical objectives that were proposed for this one-year project aimed at developing a prototype antimicrobial combat dressing. Efforts in these research tasks are expected to provide important foundational data that is expected to guide subsequent research efforts that will position this technology for commercial development of a new standard issue antimicrobial combat wound dressing.

Specific Objectives and Tasks of the Project: The following specific objectives and tasks identified for this project have been designed to allow iterative feedback enabling optimization of the copper coating process and development of a prototype antimicrobial wound dressing that may be utilized throughout the military health care system as a standard issue product.

Objective 1. Produce a candidate copper-coated dressing that balances antimicrobial efficacy and mammalian cell function, *in vitro.*

Task 1. Adjust copper coating parameters to produce a range of candidate dressings. A range of candidate copper-coated dressings will be

manufactured by altering specific coating parameters including reagent concentrations and soaking/dwell times.

Task 2. Assess the *in vitro* antimicrobial efficacy and cytotoxicity potential of candidate copper-coated dressings. Each candidate dressing will be assessed for *in vitro* antimicrobial efficacy and mammalian cell cytotoxicity potential using standardized assays that are approved by the Food and Drug Administration (FDA)

Objective 2. Assess the *in vivo* biocompatibility status of candidate copper-coated dressing. To remain in line with regulatory requirements, prototype dressings will be put forward for rigorous *in vivo* biocompatibility assessment as outlined in the below tasks, and will be conducted under Good Laboratory Practice guidelines.

Task 3. Determine the dermal irritation potential of iFyber copper-coated dressing.

Task 4. Determine the allergic response potential of iFyber copper-coated dressing.

3.2 Major Accomplishments of the Project

3.2.1 Development of a Cu-based antimicrobial wound dressing

In this initial study, iFyber focused research efforts on refining the copper coating process to provide an antimicrobial coating with a range of efficacy with respect to standard *in vitro* antimicrobial assays. As part of these efforts, iFyber has established a collaboration with Cotton Inc., the main research and marketing company representing the US cotton industry (www.cottoninc.com), to develop a chemical treatment for cotton that supports the iFyber copper coating, and is amenable to the cotton gauze wound dressing that has been selected for prototyping. Independent of coating optimization, the base prototype dressing material was selected in collaboration with our partners at H&H Associates (www.buyhandh.com), a leading vendor of standard issue military wound dressings, which is a 100% cotton gauze bearing a crinkle-weave pattern that provides a thick dressing substrate and a self-adherent property.

The chemical treatment and copper coating process is illustrated in **Figure 1**. Using a model cotton substrate for coating optimization, iFyber has refined the Cu-coating technology to provide a range of copper loadings onto the cotton substrate. An iterative process was used to establish a thorough understanding of the parameters that are important to optimizing the copper coating process (outlined below).

- **Pre-treatment to provide Anionic Cotton:** Concentration and time components for the incorporation of anionic aqueous Cu chelation chemistries to the cotton dressing are key to the development of Cu-coated cotton (e.g., anionic acetate or sulfonate groups for modified cotton surfaces). Together with our partners at Cotton Inc. who have state of the art equipment for evaluating chemical treatments of cotton, we have established a robust and

scalable process for covalently attaching acetate groups to the cotton surface. While we have also evaluated chemistries for attaching sulfonate groups to the cotton, sulfonated cotton prepared for this effort did not exhibit sufficient Cu binding properties compared to the acetate functionality. Using the acetate chemistry, we have established parameters for applying an antimicrobial Cu coating to a model substrate (outlined below), and have also established that this chemistry is compatible with the selected crinkle-weave prototype cotton-based dressing. Briefly, the process involves the steps of pad application of aqueous sodium hydroxide (NaOH), followed by pad application of chloroacetic acid and subsequent oven baking to complete the transformation to carboxymethylcellulose (i.e., cotton functionalized with acetate groups). The resulting substrate is washed to remove excess reagents and then further modified with copper as outlined below.

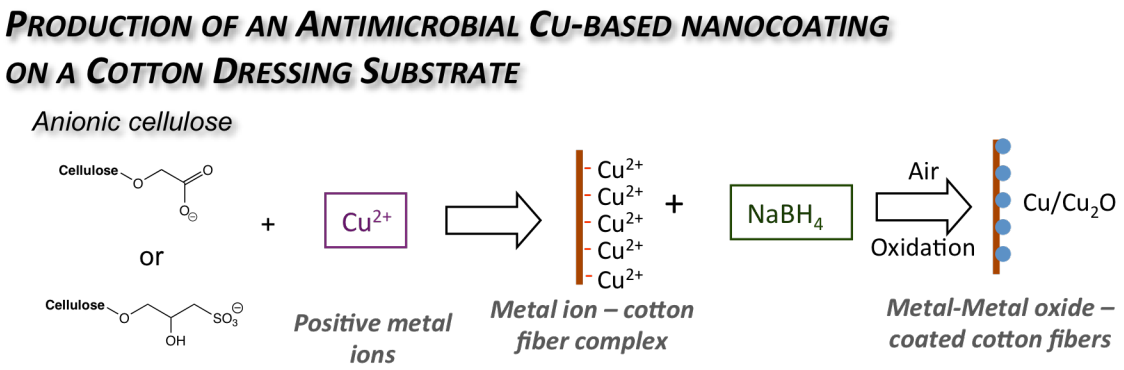


Figure 1. Schematic representation of the copper coating process used in the formation of a Cu-coated

- **Copper ion (Cu^{2+}) treatment:** The copper coating process starts with formation of a Cu^{2+}-carboxymethylcellulose complex, which is predominately controlled by the Cu^{2+} concentration and dwell time. The coating process is fairly basic and involves soaking the carboxymethylcellulose cotton in a buffered aqueous solution of Cu^{2+} (e.g., in the form of copper sulfate; $CuSO_4$) for a specified amount of time at room temperature. While we have established that Cu loading can be controlled by varying the dwell time, in the current effort, we have determined that changing the Cu^{2+} concentration provides better control over this parameter. This is largely due to the fact that Cu^{2+} binding to the cotton substrate occurs rapidly, with the majority of binding occurring in the first 5 minutes, and complete binding occurring at 30 minutes (refer to **Figure 2**; left plot).

- **Copper coating:** The final step in the process is reducing the Cu^{2+}-carboxymethylcellulose complex to a composite thin film of copper/copper oxide on the surface of the cotton fibers. The reducing agent selected for the current effort is sodium borohydride ($NaBH_4$), which we have used in previous efforts and is a common reagent used in industrial textile coating processes. We have established that complete reduction of the Cu^{2+}-carboxymethylcellulose intermediate occurs within 10 minutes to yield a Cu^{o} metal coating, which rapidly oxidizes upon drying in to yield a green composite coating of Cu^{o}/Cu_2O.

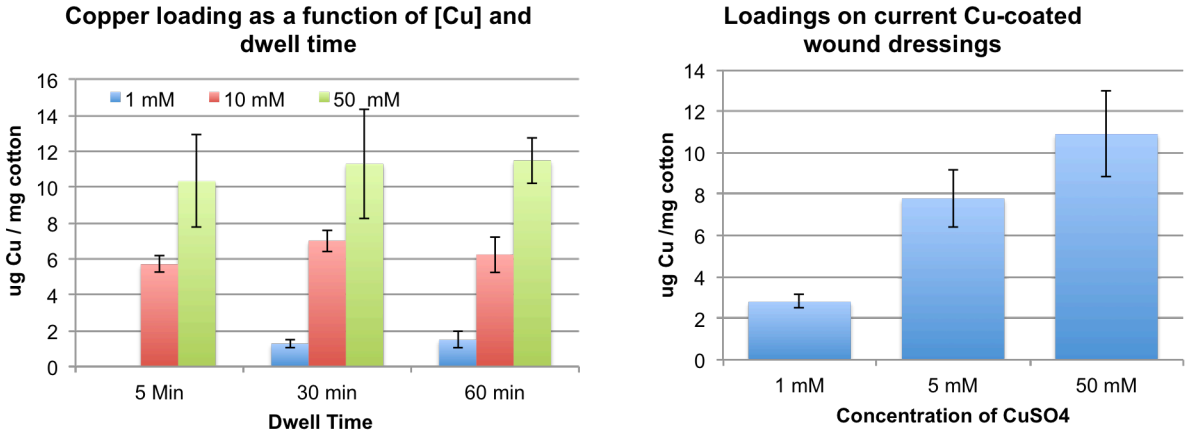


Figure 2. (left) Cu loading data for early efforts to establish the effect of Cu^{2+} concentration and dwell time on the Cu loading of Cu-coated dressings. (right) Cu loadings using established Cu coating process.

iFyber has shared the Cu coating process with researchers at Cotton Inc. and we are confident that the parameters are amenable to production at an industrial scale without the need for specialty equipment or overly burdensome processing steps. With these baseline parameters set, iFyber has evaluated the antimicrobial coating process on the prototype crinkle-weave cotton gauze, and have confirmed that this substrate will support Cu loading. **Figure 3** contains images of the cotton taken throughout the coating process starting from the unmodified native gauze. Note the faint yellow color in the anionic gauze, which is due to the carboxymethlycellulose treatment, and the characteristic faint green color of the Cu-coated gauze.

Figure 3. Photographs of the prototype cotton gauze dressing taken throughout the coating process.

Using the model cotton substrate (i.e., not gauze), iFyber also established the baseline antimicrobial performance for the Cu coating against three common wound pathogens, before and after sterilization (refer to *Sections 3.1.4* and *3.1.5*).

3.2.2 *In vitro* antimicrobial performance testing of model Cu-coated dressings

Concurrent with the selection and chemical pre-treatment of the prototype cotton gauze wound dressing, iFyber established the baseline performance metrics of the antimicrobial copper coating

using a model Cu-coated cotton substrate and a number of standard antimicrobial assays. Antimicrobial efficacy was tested against lab strains of three common wound pathogens: *Enterococcus faecalis* (ATCC 700802), *Staphylococcus aureus* (ATCC 29213), and *Pseudomonas aeruginosa* PAO1 (PAO-JP1). Antimicrobial efficacy was established relative to initial bacterial inoculum (e.g., varying colony forming units per milliliter; CFU/mL), exposure time (2-24 h), and Cu-loading (samples treated with 1 mM, 5 mM, and 50 mM Cu^{2+}; refer to **Fig 2**; right plot). Efficacy testing was done in the presence of both bacterial growth media (i.e., tryptic soy broth and agar; TSB and TSA) and in physiological buffer (i.e., phosphate buffered saline (PBS), pH 7.4) at 37°C. To date, little or no antimicrobial efficacy has been observed when growth medium is present in the assay (TSA or TSB), and although this result is noteworthy, antimicrobial agents typically fail under these conditions as these media are optimized for bacterial growth and do not represent typical conditions. Therefore, most studies reporting on the antimicrobial activity of a given substance are done in physiological buffer (with the exception of many antibiotics as these require active metabolism for efficacy). This can be done using a standard antimicrobial assay defined in ASTM method E2149-01 for determining antibacterial activity of immobilized agents under dynamic contact conditions, which is briefly described below.

The baseline antimicrobial properties for the various Cu-coated dressings described in *Section 3.2.1* were compared using a method adapted from ASTM method E2149-01. Briefly, in a 24 well culture plate, 7 mm cotton disks were placed in 1 mL of PBS and subsequently inoculated with either 10^6 or 10^8 CFU of the aforementioned bacteria. Samples were then incubated for set time periods, and the surviving bacteria in each sample well were enumerated using standard methods (serial dilution and plate counts). These data are summarized in **Figure 4**, which shows results of plate counts (represented as CFU/mL) obtained from experiments having the following variables:

- Cotton disk: Cu-coated (experimental sample), Ag^+-coated (positive control), non-coated (negative control).
- Cu-loading: reported as disks loaded with 1 mM, 5 mM, and 50 mM Cu^{2+}, which provides a differential Cu loading as reported in **Fig 2**.
- Time: contact times of 2, 6, and 24 h.
- Bacteria: *E. faecalis, S. aureus*, and *P. aeruginosa*
- Inoculum: 10^6 or 10^8 CFU

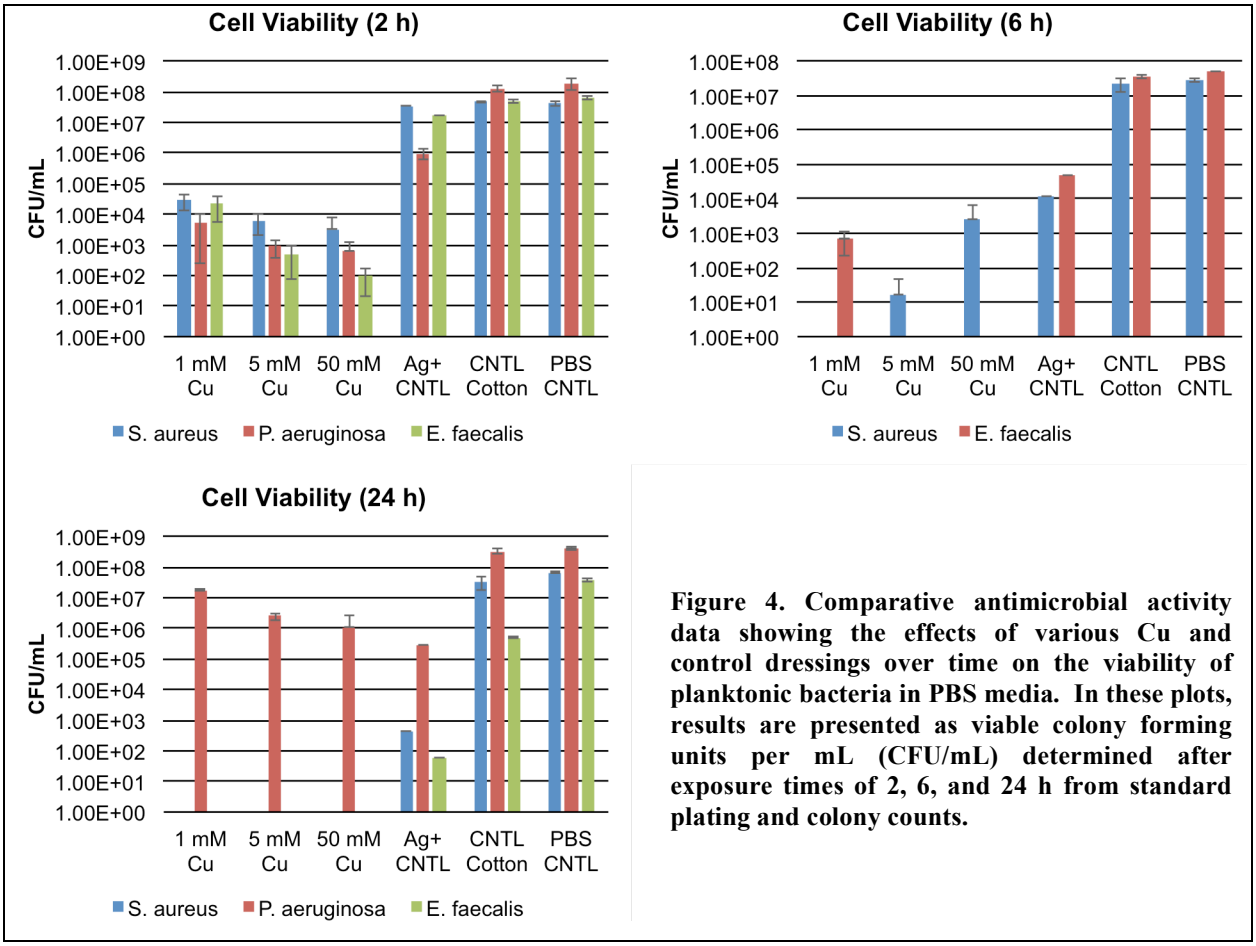


Figure 4. Comparative antimicrobial activity data showing the effects of various Cu and control dressings over time on the viability of planktonic bacteria in PBS media. In these plots, results are presented as viable colony forming units per mL (CFU/mL) determined after exposure times of 2, 6, and 24 h from standard plating and colony counts.

From the results presented in **Fig 4**, the iFyber copper coating shows excellent antimicrobial activity against the three wound pathogens tested. In general, the trends in efficacy as a function of Cu loading and time make sense (i.e., more kill with higher Cu loading and longer exposure times) with the exception of *P. aeruginosa*. Specifically, results obtained for *P. aeruginosa* show that this species is very sensitive to copper at early time points (e.g., 2 h), but is able to recover sometime after 6 hours to reach levels close to controls after 24 hours of exposure. This results is remarkable given that the cell are in an environment that should cause metabolic quiescence; however, there is literature precedent for the ability of Gram negative microbes (e.g., *E. coli*; refer to *J. Bacteriol.* 1955; 69(4): 393–398) to grow in buffer and even distilled water, presumably by feeding off dead cells and cell debris. We will continue to monitor this effect over the course of this project. Another interesting result from this assay is effect of copper on bacterial cell viability compared to silver. Although we have not yet quantified the silver loadings in the control samples, Cu-coated dressings at all loadings exhibited a greater level of efficacy than the silver dressings for all bacteria tested. Our studies also indicate that there is a dose response for the Cu-coated dressings in all species, which suggests that our coating process can be tuned to provide a range antimicrobial efficacy. This last point may be important when we begin to establish the biocompatibility profile for the coating against mammalian cells –

where we anticipate being able to tune the Cu coating to maximize antimicrobial activity while minimizing negative impacts on mammalian cells, *in vitro*.

3.2.3 *In vitro* antimicrobial activity testing against multi-drug resistant clinical isolates

The prototype Cu dressing was also tested against multidrug-resistant pathogenic microbial clinical isolates from patients, which were made available to this effort by our clinical collaborators at the State of New York Upstate Medical University. These isolates include vancomycin resistant *E. faecium* (VRE) from a penile wound, *E. coli* from biliary fluid, *P. aeruginosa* from an anterior tibia wound, Group B *Streptococcus* from penile discharge, and Methicillin-resistant *S. aureus* from an arm wound. For these studies, antimicrobial efficacy was established using a starting inoculum of ~10^6 CFU, the ASTM E2149-01 test method, and PBS as the medium. **Figure 5** summarizes the results from these studies and indicate that the prototype Cu dressings is effective against all clinical isolates, with a 4-7 log reduction in viability bacteria for a 24 hr exposure time using two Cu dressing doses (reported as total Cu content in micrograms). These results establish that the Cu dressing is a broad spectrum antimicrobial, which is expected to help in limiting nosocomial infections on the battlefield and help to prevent microbial infections in wounded soldiers. These results have been shared with our partners at H&H associates who have expressed interest in this technology as a standard issue antimicrobial combat wound dressing.

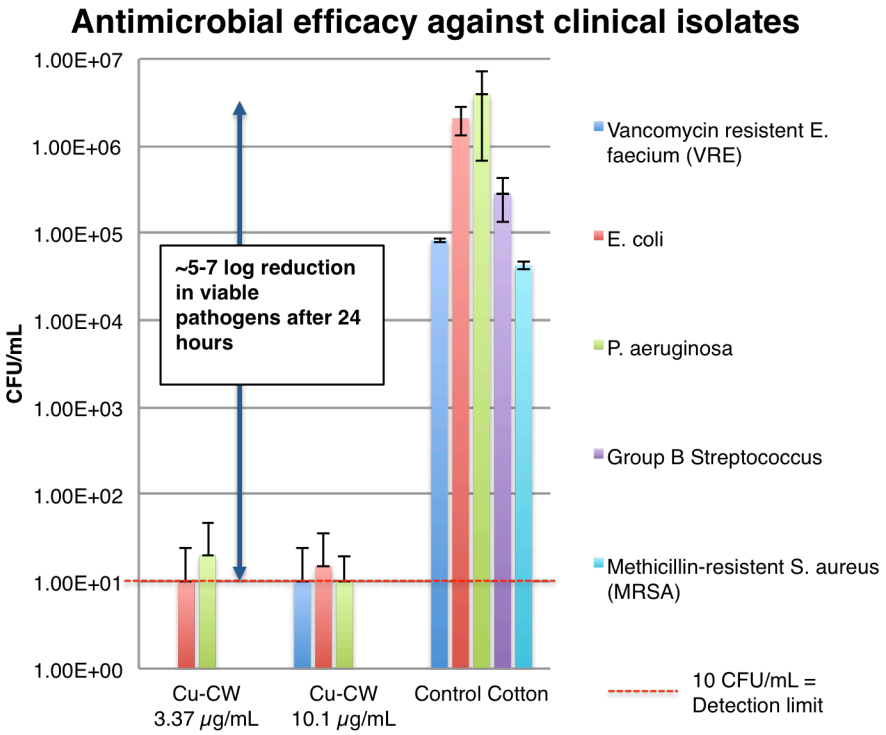


Figure 5. Results from antimicrobial activity testing against multidrug-resistant clinical isolates after 24 h exposure to two doses of Cu dressing and control dressings in PBS media.

3.2.4 Effects of sterilization on *in vitro* antimicrobial efficacy

While many marketed antimicrobial dressings do not require sterilization, we aimed to study the effect of gamma irradiated samples with respect to antimicrobial efficacy. Antimicrobial testing was done against lab strains of three common wound pathogens: *Enterococcus faecali*s (ATCC 700802), *Staphylococcus aureus* (ATCC 29213), and *Pseudomonas aeruginosa* PAO1 (PAO-JP1). Using the ASTM method E2149-01 dynamic contact assay previously described, the new batch of prototype Cu dressing showed significant antimicrobial activity against all three lab strains when the assay is conducted in PBS. **In general, no adverse effects were observed after exposure to a gamma dose of 28-30 kGy, suggesting that the end product could be sterilized if desired.**

3.2.5 *In vitro* biocompatibility testing of the prototype Cu dressing

Determining the cytotoxic status of a potential wound dressing is a key component to regulatory approval, and is governed by specific testing protocols outlined in the International Standards Organization (ISO) document 10993-5 *Biological Evaluation of Medical Devices – Tests for Cytotoxicity*. These standardized, FDA accepted assays include assessment of multiple aspects of mammalian cell function upon exposure to the test article. To remain in line with our proposed in vivo biocompatibility testing, MEM elution assays were done to determine the cytotoxic potential of the prototype Cu dressing. This approach is widely accepted for determining the cytotoxic potential of various materials that may come into contact with human tissue.

Monolayer cultures of L929 fibroblasts were propagated under standard culture parameters (37°C, 5% CO_2) in MEM media containing 10% horse serum. At 80 – 90% confluency, fibroblasts were detached from the tissue culture dish using accutase treatment and plated into 96 well tissue culture plates at a density of 10 cells/well. At the same time, dressings were extracted into cell culture media at 37°C for 24 h, after which serial dilutions of the dressing extracts were then exposed to the now semi-confluent monolayer cultures of L929 murine fibroblast cells. After a further 24 h incubation, cellular responses were quantified using the well- known MTT assay.

The MTT assay is a recognized method for quantifying **cell metabolic status** as a function of mitochondrial reductase activity. Briefly, in the MTT assay, a tetrazolium compound is introduced to cells in culture, and reduced to insoluble formazan crystals by metabolically active cells. The crystals are then solubilized by addition of a solvent (e.g., isopropanol), producing a purple solution. Results were quantified spectrophotometrically at a wavelength of 570 nm. Following guidelines laid out in ISO-10993-12 *Biological evaluation of medical devices*, 100 mg quantities of the Cu dressing were extracted for these baseline studies. Specimens were extracted with both MEM media alone and MEM media containing serum (done in triplicate), and the resulting 24 h extracts were applied to L929 cells and assessed for viability via MTT. Results from these studies are reported in **Figure 6**, and indicate that the extracts at 100% strength are toxic to cells while subsequent dilution of the extracts show significantly lower levels of toxicity

relative to controls. It is important to note that a benchmark for success, as detailed in ISO 10993-5, is maintenance of cell viability at or above 70% of control cells (i.e., vehicle only). Also important is the fact that at the 25% dilution of the extracts, which is similar in copper content (e.g., 9.9 μg total Cu) that was used in the antimicrobial efficacy studies reported in *Sections 3.2.2 – 3.2.3*, exhibits a very low level of toxicity. Taken together with the high antimicrobial activity of the dressing at this dose, these results are very promising and provide excellent baseline data for the prototype Cu dressing that were compared to the *in vivo* compatibility studies reported in *Section 3.2.6*.

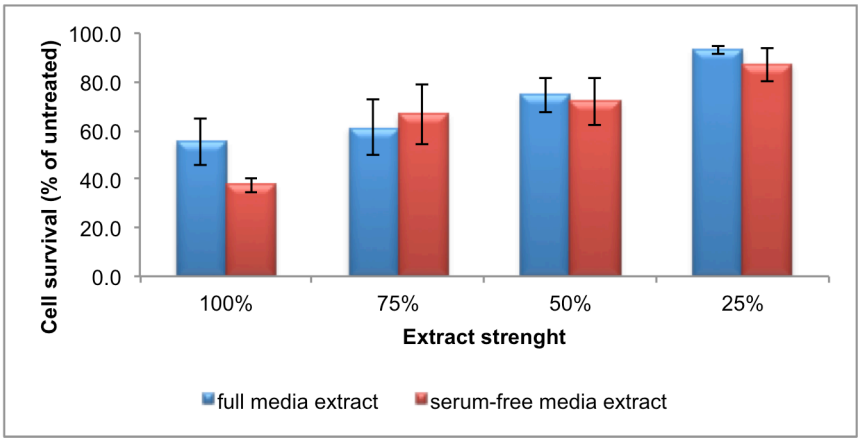


Figure 6. Results of the MTT assays showing the effect of the cotton extract on the survival of L929 cells as determined through their metabolic activity (average of three independent experiments).

3.2.6 Determination of the dermal sensitization potential of iFyber copper-coated dressing.

To determine if iFyber copper coated dressings stimulate the immune system to produce an allergic reaction, the following *in vivo* sensitization was conducted. The Guinea Pig Maximization Sensitization Test (GPMST) is an adjuvant sensitization test requiring intradermal injections of the test substance (extracted into appropriate polar and non-polar solvents) along with Freund's Complete Adjuvant (FCA), which enhances the potential of weak sensitizers by non-specifically stimulating the immune system of the animals. A group of 17 Hartley guinea pigs (11 test subjects and 6 vehicle controls) will be used in the test arm. WuXi AppTec carries out positive control testing every three months, as per their standard operating procedures. If this cycle coincides with our testing schedule, an

Table 1. Injection protocol for Induction 1.

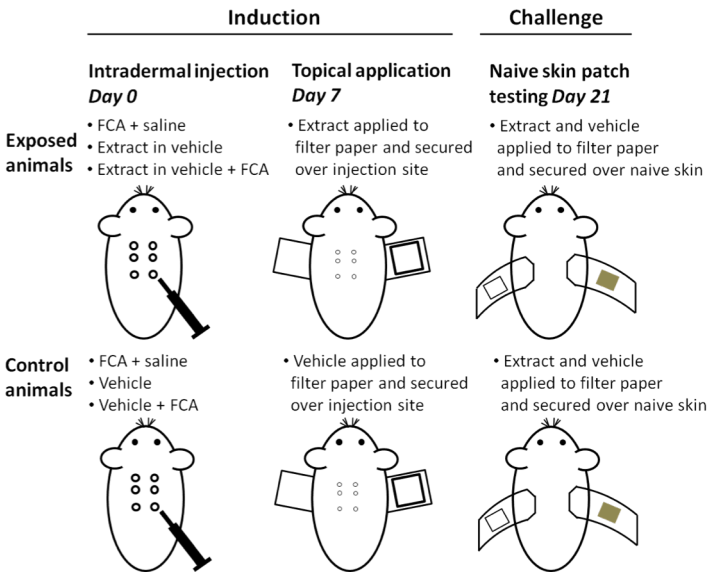
Preparation	Volume Injected Per Site	Syringe Contents	Ratio (V / V)
Test Group			
Syringe 1	0.1 mL	FCA + 0.9% Sterile Saline	1:1
Syringe 2	0.1 mL	Test Extract	NA
Syringe 3	0.1 mL	FCA + 0.9% Sterile Saline (1:1) + Test extract	1:1
Negative Control Group			
Syringe 1	0.1 mL	FCA + 0.9% Sterile Saline	1:1
Syringe 2	0.1 mL	Control Vehicle	NA
Syringe 3	0.1 mL	FCA + 0.9% Sterile Saline (1:1) + Control Vehicle	1:1

additional 17 guinea pigs will be used (11 positive control and 6 vehicle control). For positive control testing, the known sensitizer dinitrochlorobenzene (0.3%) is used, with ethanol as a vehicle. The positive control assessment will be carried out using the same procedure as used for the experimental test arm.

Induction 1 (intradermal injection). On day 0 the shoulder region of the animals will be clipped of hair, a nd three pairs of intradermal injections will be made simultaneously on either side of the midline. This is commonly referred to as Induction 1. Two solvents will be used for extraction of Cu prototype dressings, with two rows of injections being placed on either side of the midline of the animal (one polar extraction, one non-polar extraction on each side). The injection details are summarized in **Table 1**.

Induction 2 (topical application). On day 6, the injection site areas will be treated with the detergent, and known sensitizer, sodium lauryl sulfate (10% in mineral oil). On day 7, fresh Cu dressing extract will be applied to filter paper and taped over the injection site. Control animals will receive vehicle only.

Challenge phase. On day 21 the challenge procedure will be initiated on test and control animals. Fur will be clipped from both the left and right flank/dorsum region to uncover naïve skin. Freshly prepared Cu dressing extract will applied to filter paper and placed in contact with naïve skin on the right flank, and vehicle controls will be applied in a similar fashion to the left flank. Both test and control animals will receive the same treatments. The patches will be held in place by an occlusive dressing for 24 hours, after which the challenge skin areas will be observed for signs of sensitization as indicated by erythema and edema. The grading scales are detailed in the table shown in **Figure 7.**



Patch Test Reaction	Grading scale
No visible change – No erythema or edema	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and/or edema	3

Figure 7. Experimental design of the Guinea Pig maximization sensitization test (above) and associated scoring table that will be used to qualify results from this test.

Main Test Results Reported by WuXi Apptech 41923: None of the negative control animals challenged with the control vehicles were observed with a sensitization response greater than '0'. None of the test animals challenged with the

test article extracts were observed with a sensitization response greater than '0'. A negative sensitization incidence was interpreted for all test animals. See **Table 2** for individual animal scores.

Table 2. Daily Challenge Observations

Normal Saline (NS)					
Animal #	24 Hour Scores		48 Hour Scores		Results (+) or (-)
	Test Group				
	Control Vehicle	Test Extract	Control Vehicle	Test Extract	
1642	0	0	0	0	-
1644	0	0	0	0	-
1645	0	0	0	0	-
1647	0	0	0	0	-
1648	0	0	0	0	-
1649	0	0	0	0	-
1650	0	0	0	0	-
1651	0	0	0	0	-
1652	0	0	0	0	-
1654	0	0	0	0	-
1655	0	0	0	0	-
Animal #	Negative Control Group				Results (+) or (-)
	Control Vehicle	Test Extract	Control Vehicle	Test Extract	
1635	0	0	0	0	-
1636	0	0	0	0	-
1637	0	0	0	0	-
55947	0	0	0	0	-
1640	0	0	0	0	-
1641	0	0	0	0	-

Sesame Oil (SO)					
Animal #	24 Hour Scores		48 Hour Scores		Results (+) or (-)
	Test Group				
	Control Vehicle	Test Extract	Control Vehicle	Test Extract	
1662	0	0	0	0	-
1663	0	0	0	0	-
1664	0	0	0	0	-
1494	0	0	0	0	-
1672	0	0	0	0	-
1690	0	0	0	0	-
1708	0	0	0	0	-
1726	0	0	0	0	-
1746	0	0	0	0	-
1764	0	0	0	0	-
1782	0	0	0	0	-
Animal #	Negative Control Group				Results (+) or (-)
	Control Vehicle	Test Extract	Control Vehicle	Test Extract	
1656	0	0	0	0	-
1657	0	0	0	0	-
1658	0	0	0	0	-
1659	0	0	0	0	-
1660	0	0	0	0	-
1661	0	0	0	0	-

Positive Control: WuXi AppTec completes positive controls every 3 months. A positive control was completed 08/25/16 (see **Table 3** for individual animal scores). The methods for the positive control assay are similar to the methods described above in the "Experimental Summary." Guinea pigs utilized for positive control studies are of the Hartley strain and are supplied by the same vendor as animals used for general testing (Charles River Laboratories). For the Induction I and Induction II phases, a known sensitizer, 0.3% dinitrochlorobenzene (DNCB) in ethanol, was used. For the challenge phase, 0.15% DNCB in acetone was used. The negative control animals were exposed to the appropriate vehicle (ethanol was used for the Inductions I and II and acetone was used for the challenge).

At 24 hours and 48 hours after challenge patch removal, all animals in the test group were observed with discrete or patchy erythema (scores of '1'), moderate and confluent erythema (scores of '2') as well as intense erythema (scores of '3') at the challenge sites treated with 0.15% w/v DNCB in acetone. By contrast, none of the animals in the control group exhibited erythema at the challenge sites treated with 0.15% w/v DNCB in acetone (scores of '0') at either the 24-

hour or 48-hour scoring periods, with the exception of control animal #53808 which was observed with discrete or patchy erythema (score of '1') at the 24 hour scoring period. However, this was not sustained at the 48 hour scoring period, indicating that this was temporary irritation. Therefore, a 0% sensitization incidence was interpreted for the control group animals at the 48 hour scoring period. Per the evaluation criteria of the assay, the strength of the response in the test group compared to the negative control gro up indicates a sensitization response due to the repeated applications of the DNCB. Since grades of '3', '2' or '1' were observed in the test group animals at the 24 hour and 48 hour scoring periods, these represented sensitization reactions (100% sensitization incidence). Therefore, based on the results obtained, this test methodology demonstrated dermal sensitization in guinea pigs using DNCB, a known sensitizer.

Table 3. Test (ONCB) and Control Daily Challenge Observations Complete on 08/25/16

	24 Hour Scores		48 Hour Scores		Results (+) or (-)
	Test Group				
Animal #	Control Vehicle	DNCB Solution	Control Vehicle	DNCB Solution	
53809	0	3	0	2	+
53810	0	2	0	2	+
53811	0	2	0	1	+
53812	0	2	0	1	+
53813	0	3	0	2	+
53814	0	3	0	2	+
53815	0	2	0	1	+
53816	0	2	0	2	+
53817	0	3	0	2	+
53818	0	1	0	1	+
53819	0	2	0	2	+
	Control Group				Results (+) or (-)
Animal #	Control Vehicle	DNCB Solution	Control Vehicle	DNCB Solution	
53803	0	0	0	0	-
53804	0	0	0	0	-
53805	0	0	0	0	-
53806	0	0	0	0	-
53807	0	0	0	0	-
53808	0	1[a]	0	0	-

Analysis and Conclusions: None of the negative control animals challenged with the control vehicles were observed with a sensitization response greater than '0'. None of the animals challenged with the test article extracts were observed with a sensitization response greater than '0'. The normal saline extract of the test material had a sensitization response of '0' under valid test conditions. The sesame oil extract of the test material had a sensitization response of '0' under valid test conditions. **Under the conditions of this protocol, the test article did not elicit a sensitization response.**

3.2.7 Determine the allergic response potential of iFyber copper-coated dressing

To determine if iFyber copper coated dressings elicit an allergic response, the Intracutaneous Irritation Test was conducted. The New Zealand albino rabbit is the standard animal used for evaluation of potential skin irritants, and because of an extensive historical database available, allows for comparative evaluation of products. Three New Zealand albino rabbits (≥2.0 kg) were used for the study.

The Intracutaneous Irritation Test is conducted much like allergy testing in human patients. Fluid extracts of the test material are prepared under controlled conditions of temperature, time, and ratio of the material surface area to the volume of extraction fluid. The following two extraction vehicles and extraction parameters were chosen based on recommended ISO guidelines (ISO 10993-10: *Tests for irritation and delayed-type hypersensitivity*); 0.9% normal saline and sesame

oil. iFyber dressing samples were extracted a ratio of 0.1 g / 1 mL of extraction vehicle. The extraction mixtures and corresponding control blanks were incubated for 72 ± 2 hours at 50 ± 2 C. At the start of the extraction, the solutions appeared clear and free of particulates. The extracts were agitated during the course of the extraction period. At the end of the extraction period, the vessels were shaken well and the liquid aseptically decanted into a sterile vessel. The extracts were not further manipulated prior to use. The extracts were maintained at room temperature and used within 24 hours of preparation.

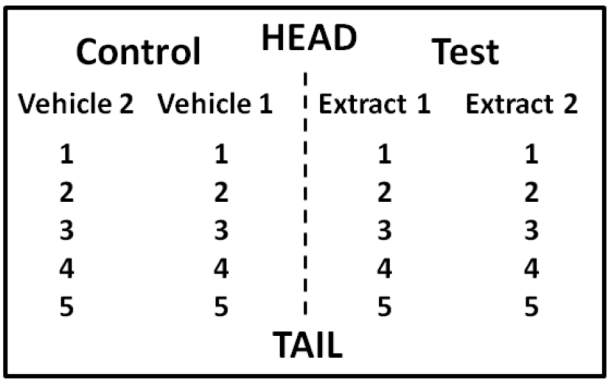


Figure 5. Diagrammatic representation of extract/vehicle injection sites on dorsal region of rabbits.

Using small-bore needles, 0.2 mL of fresh extract was injected intracutaneously at five sites on the right side of the spinal column. The corresponding vehicle controls were also injected at five sites on the left side of the spinal column. The second extract was injected on sites parallel and distal to the test and control injections. (see diagrammatic representation in **Figure 5**). The appearance of each site was observed immediately post-injection and at 24, 48, and 72 hours post injection. The tissue reactions were then evaluated and scored according to **Table 2** for gross evidence of erythema, edema, and necrosis.

Dermal Irritation Results:

Clinical Observations. None of the animals on study showed abnormal clinical signs during the 24, 48, and 72 hour observation periods.

Dermal Observations. There were no significant dermal reactions observed at the injected test and control sites on the rabbits at the 24, 48, and 72 hour observation periods.

Calculations. After the 72 ± 2 hour observation period, all erythema grades plus edema grades 24 ± 2, 48 ± 2, and 72 ± 2 hour were totaled separately for each test sample or control for each individual animal. To calculate the score of a test sample or control on each individual animal, each ofthe totals was divided by 15 (three scoring time points x five test or control sample injection sites). To determine the overall mean score for each test sample and each corresponding control, the scores were added for the three animals and divided by three. The final test sample score was obtained by subtracting the score of the control from the test sample score. The results are presented in Tables 6-7.

Positive Control. A positive control was completed prior to the start of testing the iFyber samples – NOTE: WuXi AppTec completes positive controls at least every 6 months as required per ISO guidelines. The methods for the positive control assay are performed similar to the Experimental Summary above, except that the test and control solutions are dosed neat and not

extracted. A solution of 0.15% sodium lauryl sulfate (dissolved in 0.9% normal saline) is used as the test solution and 0.9% normal saline is used as the control. After the 72 hour test period all rabbits elicited a positive reactivity response and the comparative result of the positive control assay was greater than 1.0, indicating a positive response.

Analysis and Conclusions. The test was considered valid based upon scientific judgment. Results for the saline extract and sesame oil extract are shown in **Tables 4** and **5**, respectively. The differences in the mean test and control scores of the extract dermal observations were less than 1.0, indicating that the requirements of the ISO Intracutaneous Reactivity Test have been met by the test article, and thus, the iFyber copper coatings elicit little potential for dermal irritation.

Table 4. Dermal Observations – 0.9% Normal Saline

Rabbit # 49377	Control Scores						Test Scores					
	24 Hour		48 Hour		72 Hour		24 Hour		48 Hour		72 Hour	
	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED
Site 1	0	0	0	0	0	0	0	0	0	0	0	0
Site 2	0	0	0	0	0	0	0	0	0	0	0	0
Site 3	0	0	0	0	0	0	0	0	0	0	0	0
Site 4	0	0	0	0	0	0	0	0	0	0	0	0
Site 5	0	0	0	0	0	0	0	0	0	0	0	0
Total	0		0		0		0		0		0	
Rabbit # 49378	**Control Scores**						**Test Scores**					
	24 Hour		48 Hour		72 Hour		24 Hour		48 Hour		72 Hour	
	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED
Site 1	0	0	0	0	0	0	0	0	0	0	0	0
Site 2	0	0	0	0	0	0	0	0	0	0	0	0
Site 3	0	0	0	0	0	0	0	0	0	0	0	0
Site 4	0	0	0	0	0	0	0	0	0	0	0	0
Site 5	0	0	0	0	0	0	0	0	0	0	0	0
Total	0		0		0		0		0		0	
Rabbit # 49379	**Control Scores**						**Test Scores**					
	24 Hour		48 Hour		72 Hour		24 Hour		48 Hour		72 Hour	
	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED
Site 1	0	0	0	0	0	0	0	0	0	0	0	0
Site 2	0	0	0	0	0	0	0	0	0	0	0	0
Site 3	0	0	0	0	0	0	0	0	0	0	0	0
Site 4	0	0	0	0	0	0	0	0	0	0	0	0
Site 5	0	0	0	0	0	0	0	0	0	0	0	0
Total	0		0		0		0		0		0	
Rabbit #	**Control Scores (Total ER & ED)**						**Test Scores (Total ER & ED)**					
49377	0		0		0		0		0		0	
49378	0		0		0		0		0		0	
49379	0		0		0		0		0		0	
Rabbit #	**Total / 15**						**Total / 15**					
49377	0						0					
49378	0						0					
49379	0						0					
Average (Total / 3)	0 / 3 = 0						0 / 3 = 0					
Comparative Results (Average Test – Average Control)	0 – 0 = 0											

ER=Erythema ED=Edema

Table 5. Dermal Observations – Sesame Oil

Rabbit # 49377	Control Scores						Test Scores					
	24 Hour		48 Hour		72 Hour		24 Hour		48 Hour		72 Hour	
	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED
Site 1	1	0	1	0	1	0	2	0	1	0	1	0
Site 2	1	0	1	0	1	0	1	0	1	0	1	0
Site 3	1	0	1	0	1	0	2	0	1	0	1	0
Site 4	1	0	1	0	1	0	2	0	1	0	1	0
Site 5	1	0	1	0	1	0	2	0	2	0	1	0
Total	5		5		5		9		6		5	
Rabbit # 49378	**Control Scores**						**Test Scores**					
	24 Hour		48 Hour		72 Hour		24 Hour		48 Hour		72 Hour	
	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED
Site 1	1	0	1	0	1	0	1	0	1	0	1	0
Site 2	1	0	1	0	1	0	1	0	1	0	1	0
Site 3	1	0	1	0	1	0	1	0	1	0	1	0
Site 4	1	0	1	0	0	0	1	0	1	0	1	0
Site 5	1	0	1	0	1	0	1	0	1	0	0	0
Total	5		5		4		5		5		4	
Rabbit # 49379	**Control Scores**						**Test Scores**					
	24 Hour		48 Hour		72 Hour		24 Hour		48 Hour		72 Hour	
	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED	ER	ED
Site 1	1	0	1	0	1	0	1	0	1	0	1	0
Site 2	1	0	1	0	0	0	1	0	1	0	1	0
Site 3	1	0	1	0	0	0	1	0	1	0	1	0
Site 4	1	0	1	0	0	0	2	0	1	0	1	0
Site 5	1	0	1	0	0	0	1	0	1	0	1	0
Total	5		5		1		6		5		5	
Rabbit #	**Control Scores (Total ER & ED)**						**Test Scores (Total ER & ED)**					
49377	5		5		5		9		6		5	
49378	5		5		4		5		5		4	
49379	5		5		1		6		5		5	
Rabbit #	**Total / 15**						**Total / 15**					
49377	1.0						1.3					
49378	0.9						0.9					
49379	0.7						1.1					
Average (Total / 3)	2.6 / 3 = 0.9						3.3 / 3 = 1.1					
Comparative Results (Average Test – Average Control)	1.1 – 0.9 = **0.2**											

ER=Erythema ED=Edema

3.3 Project Status after Year 2 Performance Period

The following table is used to help track the status of the project relative to the goals stated in *Section 3.1* and the major technical objectives and tasks that are needed to achieve these goals. For each technical objective the overall status of the work is qualified, and the major findings are listed.

Technical Objective 1 – Produce prototype copper-coated dressings that balance antimicrobial efficacy and mammalian cell function, *in vitro.*	**Overall Status**	**Major Findings (Q1)**	**Timeline for Completion**
Tasks 1 and 2			
Subtask 1: Establish coating parameters that produce	**Complete**	• Established cotton pre-treatment	**Complete**

candidate dressings that offer maximal antimicrobial action without comp-romising mammalian cell viability.	Baseline coating parameters are set and will allow coating adjustments to be made to maximize antimicrobial performance and minimize effects to mammalian cells, *in vitro*	process • Established important parameters for changing Cu loadings • Established Cu coating on prototype gauze dressing	**Q1**
Subtask 2: Establish baseline antimicrobial performance metrics for copper-coated candidate dressings prepared in Subtask 1.	**Complete** Baseline performance has been established for 3 wound pathogens as well as for 5 mulidrug-resistant clinical isolates.	• Cu coatings effective against 3 common wound pathogens and 5 multidrug-resistant clinical isolates • 4-7 log reduction in viable bacteria can be achieved within 2 hr of exposure.	**Complete**
Subtask 3: Establish baseline biocompatibility performance metrics for copper-coated candidate dressings prepared in Subtask 1.	**Complete** Baseline cytotoxicity profile has been established for the prototype Cu dressing	• The Cu coating shows some cytotoxicity potential at high loading, but is within FDA accepted criteria at the loadings used to establish antimicrobial activity.	**Complete**
Subtask 4: Conduct antimicrobial assays on sterilized samples to determine if sterilization alters dressing performance.	**Pending** (Q4)		**Complete**
Technical Objective 2 – Establish the biocompatibility profile of an optimized prototype copper-coated dressing, *in vivo.*			
Tasks 3 and 4			

Subtask 1: Obtain IACUC and USAMRMC ACURO approvals for animal studies to be performed in Subtasks 2-3 below.	**Complete**		**Complete**
Subtask 2: Assess the dermal irritation potential of the prototype copper-coated dressing.	**Complete**	• Cu dressing did not elicit a dermal irritation effect	**Complete**
Subtask 3 Assess the allergic potential of the sterilized copper-coated prototype dressing.	**Complete**	• Cu dressings did not elicit a sensitization effect	**Complete**

4.0 Impact

Nothing to Report

5.0 Changes/Problems

Nothing to Report

6.0 Products

Nothing to Report

7.0 Participants and Other Collaborating Organizations

Name:	Aaron D. Strickland
Project Role:	Principal Investigator / Project Manager
Nearest person month worked:	1
Contribution to Project:	Dr. Strickland has been responsible for all experimental designs and has participated in data analysis and interpretation of results. Dr. Strickland has organized communication with partners and H&H Associates and Cotton Inc.

Name:	Deborah Diehl
Project Role:	Research Scientist (Biology)
Nearest person month worked:	1
Contribution to Project:	Mrs. Diehl has been responsible for conducting the bulk of the antimicrobial assays and has also participated in the production and chemical analysis of the model Cu wound dressings.

Name:	Alison Schug
Project Role:	Technician
Nearest person month worked:	1
Contribution to Project:	Ms. Schug has performed work on the production and assessment of metal loading content of the model Cu wound dressings.

Name:	Nina Bionda
Project Role:	Sr. Research Scientist (Biology)
Nearest person month worked:	1
Contribution to Project:	Dr. Bionda collaborated with Dr. Strickland to manage the day to day laboratory activities and design of experiments. Dr. Bionda has also led the *in vitro* biocompatibility efforts.

Name:	Matt Farrell, PhD
Project Role:	Manager, Textile Chemistry Research at Cotton, Inc.
Nearest person month worked:	1
Contribution to Project:	Dr. Farrell works for Cotton Inc, and collaborated with Dr. Strickland to produce anionic cotton dressing material for the attachment of iFyber Cu coating on cotton.

Name:	Joseph DeCorta
Project Role:	Vice President at H&H Medical, Inc.
Nearest person month worked:	1
Contribution to Project:	Mr. DaCorta is a medical device expert and has help to guide iFyber efforts towards a relevant cotton-based standard issue combat dressing current offered by H&H.

8.0 SECTION 5 – Special Reporting Requirements

Quad chart attached

A Nanolayer Copper Coating for Prevention of Nosocomial Multi-drug Resistant Infections
ERMS/Log Number 12117004
Award Number: W81XWH-15-2-0066

PI: Aaron D. Strickland, PhD **Org:** iFyber, LLC **Award Amount:** $114,830

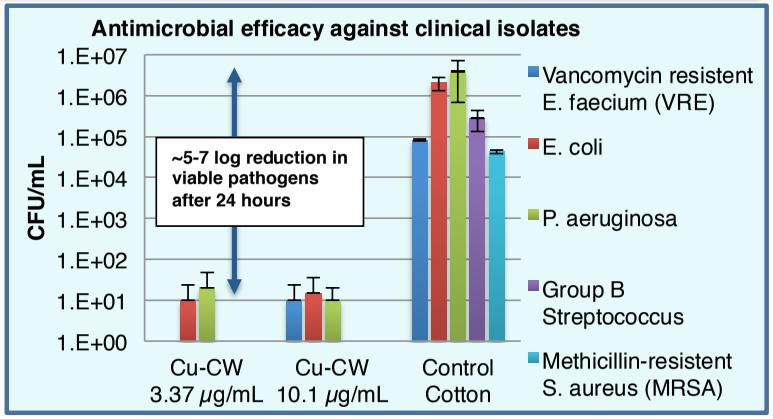
Study/Product Aim(s)

Aim 1. Produce a candidate copper-coated dressing that balances antimicrobial efficacy and mammalian cell function, *in vitro.*

Aim 2. Assess the in vivo biocompatibility status of candidate copper-coated dressing.

Approach

The immediate research and development goals set forth in the current effort are centered on identifying and optimizing an antimicrobial and biocompatible copper coating for a standard issue wound dressing. The process for producing copper coated dressings will be optimized and tested in a battery of *in vitro* antimicrobial and mammalian cell viability assays, with a final assessment of biocompatibility coming from *in vivo* studies.



Accomplishment: iFyber's prototype Cu wound dressing exhibits significant anti-microbial activity against multidrug-resistant pathogens isolated from clinical patients.

Timeline and Cost

Activities	Q1	Q2	Q3	Q4
Prototype dressing (Product/Opt)				
Prototype dressing (Efficacy/Tox)				
In vivo biocomp. (Dermal irritation)				
In vivo biocomp. (Allergic potent.)				
Estimated Budget ($K)	**$28.7K**	**$28.7K**	**$28.7K**	**$28.7K**

Updated: (August 9, 2017)

Goals/Milestones

Production and testing of model dressings

☑ Production of dressings having a range of copper loadings
☑ Established antimicrobial efficacy of copper coated dressings

***In vitro* Antimicrobial and Biocompatibility Testing**

☑ Assess activity of dressings against clinically isolated wound pathogens
☑ Establish biocompatibility profile of Cu dressings, *in vitro*
☑ *Dressings showed acceptable toxicity profile with potent antimicrobial activity*

In vivo biocompatibility

☑ Assess activity of dressings against clinically isolated wound pathogens
☑ Establish biocompatibility profile of Cu dressings, *in vitro*
☑ *Cu dressings do not elicit a sensitization or irritation response.*

Comments/Challenges/Issues/Concerns

- Nothing to report

Budget Expenditure to Date

Projected Expenditure: $114,830
Actual Expenditure: $114,830 (as of August 9, 2017)